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Effects of lipopolysaccharide on gene expression of antimicrobial peptides (penaeidins and crustin), serine proteinase and prophenoloxidase in haemocytes of the Pacific white shrimp, *Litopenaeus vannamei*

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Abstract

For shrimp immune defences, prophenoloxidase (proPO) activating system and antimicrobial peptides in circulating haemocytes play important roles. In the present study, the effects of lipopolysaccharide (LPS) injection on gene expression of penaeidins, crustin, serine proteinase and proPO in haemocytes were determined using real-time reverse transcription–polymerase chain reaction (PCR) in the Pacific white shrimp *Litopenaeus vannamei*. After injection of LPS, mRNA levels of antimicrobial peptides, penaeidin 2 (PEN2), penaeidin 3 (PEN3), penaeidin 4 (PEN4) and crustin decreased in a dose-dependent manner, while mRNA levels of serine proteinase and proPO did not change significantly. In a time-course experiment, injection of LPS caused significant depression in mRNA levels of PEN2, PEN3, PEN4 and crustin at 4 h post-injection, and the depressed mRNA levels returned to initial levels by 72 h post-injection. On the other hand, mRNA levels of serine proteinase and proPO did not show a significant change after injection. These results suggest that the antimicrobial peptide system responds to LPS injection at a gene expression level while the proPO system does not respond at a gene expression level.

Keywords: Crustin; Innate immunity; Lipopolysaccharide; mRNA; Penaeidins; proPO cascade; Real-time polymerase chain reaction; Serine proteinase; Shrimp

1. Introduction

For immune defence, shrimps, as other invertebrate species, have an innate immune system, which is mainly mediated by circulating haemocytes [1–3]. The first process of immune defence is to detect invading microorganisms such as viruses, bacteria and fungi, and the detecting mechanisms recognize cell wall components from

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microorganisms such as lipopolysaccharide (LPS), peptidoglycan and β-1,3-glucans [4,5]. Following the non-self recognition, defence systems such as phagocytosis, melanization, encapsulation and lysis of foreign cells are activated [1,4], and the prophenoloxidase (proPO) activating system and antimicrobial peptides play important roles in immune defence systems [5,6].

For the non-self recognition system, several pattern-recognition proteins such as β-1,3-glucan binding protein, LPS binding protein, peptidoglycan binding protein, and LPS and β-1,3-glucan binding protein, have been reported in several crustacean species including penaeid shrimps [4,5], and they activate the defence system after binding to the cell wall components from microorganisms [4,5]. The non-self recognition system of crustaceans has been examined for the LPS-mediated haemolymph coagulation cascade in the horseshoe crab, *Tachypleus tridentatus* as a model [7,8]. In the horseshoe crab, the circulating haemocytes respond to LPS, a major cell wall component of Gram-negative bacteria, and secrete various defence molecules (i.e. coagulation factors such as factor C, protease inhibitors, antimicrobial substances, and lectins), resulting in the initiation of the coagulation cascade [7,8]. The factor C, an LPS-recognizing serine protease zymogen that initiates the coagulation cascade, also occurs on the haemocyte surface and functions as a biosensor for LPS [9].

In proPO activating system, an inactive form of proPO is synthesized in haemocytes and converted to an active form of phenoloxidase (PO) by a serine proteinase, and PO promotes the melanization and the production of reactive oxygen species for immune defences [5,10]. Since the first cloning of proPO in the crayfish *Pacifastacus leniusculus* [11], proPO has been cloned in several crustacean species [5] including some penaeid shrimps and its gene expression in haemocytes determined [12–14]. On the other hand, a serine proteinase was cloned in several crustacean species including some penaeid shrimps [15–18], and based on its similarity in structure to insect serine proteinases, it acts to convert proPO to PO [5,18]. The proPO activating system is stimulated by LPS and β-1,3-glucans through the non-self recognition system [10,11,18].

Antimicrobial peptides that have been found in many species of vertebrates and invertebrates are key factors of innate immunity in penaeid shrimps [6], and penaeidins and crustins have been reported as antimicrobial peptides in penaeid shrimps [19,20]. Penaeidins, initially isolated from the Pacific white shrimp *Litopenaeus vannamei* (Boone) [19], are a family of antimicrobial peptides that have been detected in several penaeid shrimps [6]. Penaeidins are 5.5–6.6 kDa peptides with a N-terminal proline-rich domain and a C-terminal domain containing six cysteine residues and have antimicrobial activity against Gram-positive bacteria and fungi [19,21]. Furthermore, an opsonic property for penaeidins has been shown in in vitro confrontation of *L. vannamei* haemocytes with *Vibrio alginolyticus* [21]. Penaeidins have several isoforms and are classified into penaeidin 2 (PEN2), penaeidin 3 (PEN3), and penaeidin 4 (PEN4) according to their similarity of amino acid sequence [22]. The classification and characterization of penaeidin isoforms have been summarized in the database, PenBase [23]. In the present study, the nomenclature for penaeidins in PenBase is adopted. Between the three penaeidin subgroups, PEN3 is the most abundant at both levels of peptide and mRNA in *L. vannamei* haemocytes [19,24]. Crustin was first isolated from the shore crab *Carcinus maenas* and characterized as a cysteine-rich 11.5-kDa peptide with antimicrobial activity against Gram-positive bacteria [25]. The presence of homologues of crustin was determined in *L. vannamei* and the Atlantic white shrimp *Litopenaeus setiferus* [20], and penaeid crustins were further divided into crustins I and P [26]. These antimicrobial peptides are dominantly synthesized and stored in haemocytes [20,24,26], and their release from haemocytes is induced by bacterial infection [6,21].

Following the cloning of these molecules, gene expression analysis has been performed in penaeid shrimps, and it has been revealed that inoculation of *Vibrio* reduces mRNA levels of PEN3 [21,24] and crustin I [26] but does not affect mRNA levels of a serine proteinase [18] in *L. vannamei* haemocytes. Furthermore, it has been demonstrated that oral administration of peptidoglycan induces a marked increase in mRNA levels of crustin and serine proteinase in the kuruma prawn, *Marsupenaeus japonicus* [27,28]. However, the effect of LPS on gene expression of these molecules has not been clarified so far.

The main aim of the present study was to determine the effects of LPS injection on gene expression of penaeidins, crustin, serine proteinase and proPO in *L. vannamei* haemocytes. For this purpose, dose–response and time-course changes of their mRNA levels after LPS injection were investigated. An additional aim of the present study was to confirm the feasibility of use of real-time PCR for monitoring shrimp immune state in gene expression levels. Real-time PCR is a sensitive, rapid and robust method to quantify mRNA levels [29] and is potentially useful for a monitoring purpose.